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The histone variant macroH2A1.1 is recruited to DSBs through a mechanism involving PARP1

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ABSTRACT

The repair of DNA double-strand breaks (DSBs) requires remodeling of the local chromatin architecture to allow the repair machinery to access sites of damage. Here, we report that the histone variant macroH2A1.1 is recruited to DSBs. Cells lacking macroH2A1 have defective recruitment of 53BP1, defective activation of chk2 kinase and increased radiosensitivity. Importantly, macroH2A1.1 is not incorporated into nucleosomes at DSBs, but instead associates with the chromatin through a mechanism which requires PARP1 activity. These results reveal an unusual mechanism involving a direct association of macroH2A1.1 with PARylated chromatin which is critical for retaining 53BP1 at sites of damage.

1. Introduction

The repair of DSBs is a complex process involving activation of the ATM kinase and loading of DNA repair proteins onto the chromatin at the site of damage. Consequently, DSB repair requires significant remodeling of the chromatin to facilitate access to sites of DNA damage and to promote processing and repair of the DNA [1,2]. This reorganization of the chromatin landscape involves both chromatin remodeling ATPases [1,3] as well as histone modification, including acetylation of histone H4 by the Tip60 acetyltransferase [3,4] and ubiquitination of the chromatin by the RNF8 and RNF168 ubiquitin ligases [5]. These modifications function together to create open chromatin structures at sites of damage and to provide binding sites to recruit the brca1 complex and other proteins involved in DSB repair [2].

In addition to histone modification, chromatin structure may be regulated by the exchange of histone variants onto nucleosomes [6]. macroH2A1 is a H2A variant originally identified as a component of the inactive X-chromosome but which also controls gene expression, heterochromatin formation and cell cycle progression [7,8]. macroH2A contains a large globular macrodomain at the c-terminal which may function to bind PAR and associated metabolites [9]. PAR is a post-translational modification created at DNA strand breaks by the PARP1 enzyme [10,11]. Several macrodomain containing proteins, including Alc1, are recruited to sites of DNA damage through interaction between their macrodomains and PAR on the chromatin [9,12,13]. Macrodomains therefore represent a potential module for the PARP1 dependent loading of DNA repair proteins at sites of DNA damage [9]. Here, we examined the role of macroH2A1 in regulating chromatin structure during DSB repair.

2. Materials and methods

2.1. Cells

Culture of U2OS, HeLa and 293T cells and irradiation are described in [3,14,15]. Lentiviral shRNA plasmids targeting mH2A1 (CCAGTTACTTCGTGTCTACAA; Broad Institute, Cambridge MA)

Abbreviations: ChIP, chromatin immunoprecipitation; BER, base excision repair; DSB, DNA double-strand break; HR, homologous recombination; mH2A1, macroH2A1; IF, immunofluorescence; IR, ionizing radiation; MMS, methylmethane sulfonate; MRN, Mre11-Rad50-Nbs1; NHEJ, non-homologous end-joining; PAR, poly-ADP-ribose; PBS, phosphate buffered saline; RT-qPCR, real time quantitative PCR; ZFN, zinc finger nuclease

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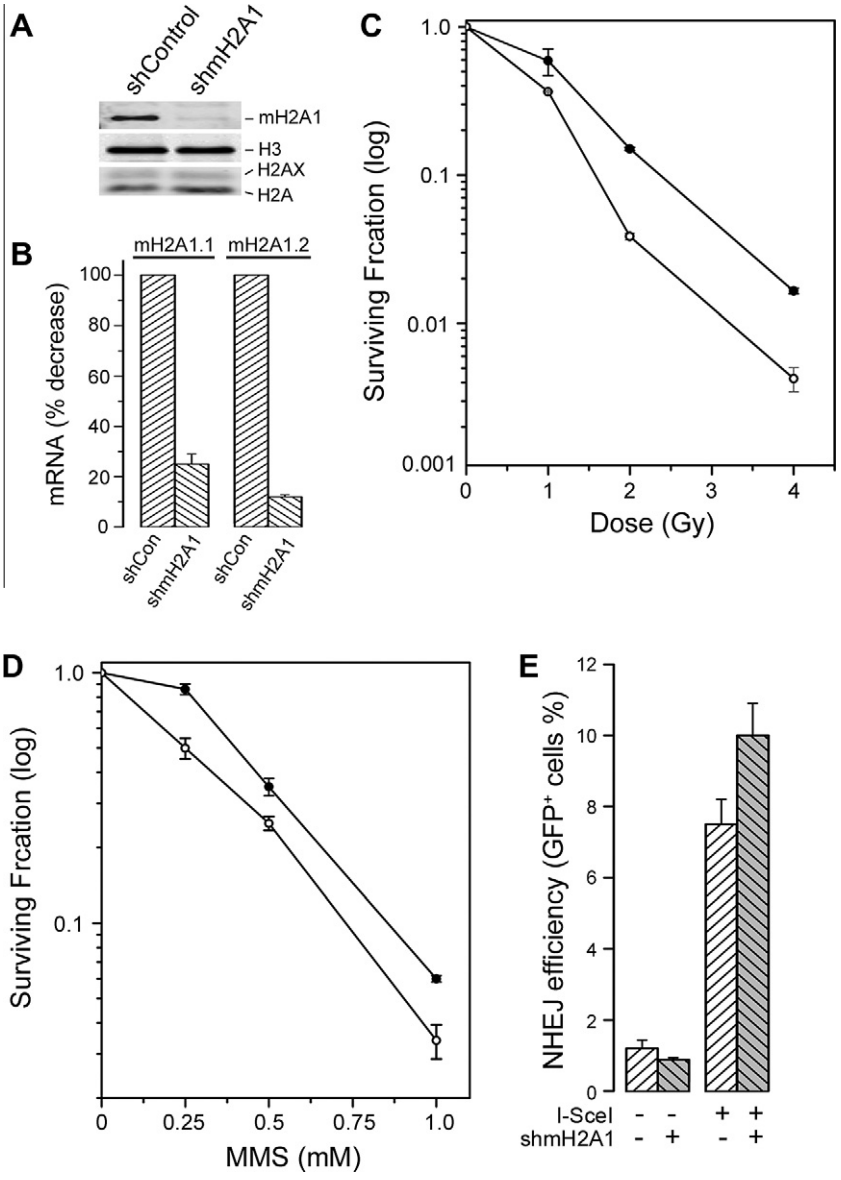

Fig. 1. Depletion of macroH2A1 increases radiosensitivity. (A) 293T cells were infected with a non-specific shRNA (shCon) or shRNA targeting mH2A1.1 or mH2A1.2 (shmH2A1). mH2A1 was measured by western blot. (B) mH2A1.1 and mH2A1.2 mRNA levels were measured by RT-qPCR in shCon and shmH2A1 cells. (C) and (D) 293T cells expressing a non-specific shRNA (shCon: ●) or shmH2A1 (○) were irradiated (C) or incubated with MMS for 1 h (D). Clonogenic cell survival was measured 10 days later. Results ± SE (n = 3). (E) HeLa cells with an integrated NHEJ-GFP reporter were stably infected with control shRNA or shRNA targeting mH2A1. I-SceI was transiently expressed and GFP$^+$ cells counted by FACS 48hr later. Results ± SD (n = 3).

were transfected as in [16]. HeLa cells expressing the NHEJ reporter [17] were transfected with I-SceI using Lipofectamine 2000 (Invitrogen, CA) and GFP positive cells counted using a BD FACscan cell analyzer with the Flow Jo software package. For clonogenic cell survival, cells were plated in triplicate, irradiated and allowed to recover for 10–14 days. Cells were fixed and stained with crystal violet and colonies with > 50 cells were scored visually as previously described [14]. Surviving fraction was calculated as [Colonies + IR]/[Colonies−IR] for each individual cell line.

2.2. ChIP assays

293T cells were transfected with p84-ZFN as described [3]. ChIP utilized the SimpleChIP™ Enzymatic Chromatin IP Kit (Cell Signaling Technology, MA) described in [3]. Cells were fixed in formaldehyde (1%), lyzed in ChIP buffer and sonicated (Fisher Scientific Sonic 250). Input DNA was digested with proteinase K and quantitated by RT-qPCR. Sonicated chromatin was incubated with primary antibody (2 h at 4 °C), followed by protein G agarose beads precoated with sperm DNA. Following washing in low and high salt ChIP buffers, samples were eluted, incubated in NaCl (65 °C for 2 h) and digested with proteinase K. DNA was quantitated by RT-qPCR using the StepOnePlus real time PCR system (Applied Biosystems, CA). Primers and amplification procedures are in Supplementary methods.

2.3. Western blot analysis

Cells were lyzed in 100 mM Tris pH6.8, 20% Glycerol, 200 mM DTT, 4% SDS, 0.01% bromophenol blue. Proteins were separated by SDS–PAGE and analyzed by western blot. Antibodies are listed in Supplementary methods.

2.4. Immunofluorescence analysis

Cells were cultured on cover slips, irradiated, washed in PBS and fixed in PBS containing paraformaldehyde (2%). Subsequently, cells were blocked with serum (2%) and permeabilized in Bovine Serum Albumen (0.2%) containing saponin (0.2%) for 10 min. Following incubation with primary antibody for 1 h, cells were washed and incubated with secondary antibody for 1 h. Slides were mounted with Fluoromount-G (Southern Biotech, AL) and images collected with a Zeiss AxioImager Z1 microscope equipped with an Axiocam MRc Rev.3 Color Digital Camera and a Plan APO 63X/1.4 oil M27 lens (magnification 63X, aperture 1.4). Image processing was carried out with the Zeiss AxioVision software package (Zeiss Imaging, NY). Images were obtained for at least 100 cells per experimental point. Cells were visually inspected, with nuclei containing > 5 foci scored as positive. All samples were examined by 2 independent workers to eliminate bias.

3. Results

mH2A1 encodes 2 splice variants, mH2A1.1 and mH2A1.2 [7]. These splice variants differ in that mH2A1.1 contains a functional macrodomain whereas mH2A1.2 is deficient in binding of ADP-ribose [9]. We identified a single shRNA which targeted both splice variants and depleted both mH2A1.1 and mH2A1.2 protein (Fig. 1A). Because the antibody does not distinguish mH2A1.1 and mH2A1.2, we also measured mRNA levels to ensure both isoforms were depleted by the shRNA (Fig. 1B). To determine how mH2A1.1 and mH2A1.2 (referred to as mH2A1 hereafter) impact DNA repair, we examined sensitivity to IR, which creates DNA strand breaks, and MMS, which methylates DNA. Depletion of mH2A1 increased sensitivity to both IR (Fig. 1C) and MMS (Fig. 1D), indicating a role for mH2A1 in the DSB and base excision repair pathways. To detail how mH2A1 contributes to DSB repair, we used a GFP based reporter system [17] to monitor NHEJ activity. Loss of mH2A1 led to a significant increase in NHEJ activity (Fig. 1E), indicating that mH2A1 may function to restrain NHEJ.

Next, we examined how loss of mH2A1 recruitment impacted the cells response to DSBs. The kinetics of H2AX phosphorylation were relatively unaltered in mH2A1 depleted cells (Fig. 2A; Supplementary Fig. 1A), although there was a statistically significant reduction in γH2AX levels at 30 min. However, activation of ATM (Fig. 2B) and the recruitment of the rad51 protein (Supplementary Fig. 1B) to DSBs were not affected by loss of mH2A1. Recruitment of mH2A1 to DSBs does not, therefore, impact the early signaling events activated in response to DSBs. We also examined 53BP1, a DSB repair protein which plays an essential role in regulating NHEJ [18,19] and is important for modulating the ability of ATM to phosphorylate its target proteins [20,21]. Surprisingly, cells lacking mH2A1 displayed a significant inhibition of 53BP1 accumulation (Fig. 2C; Supplementary Fig. 1A), implicating mH2A1 in recruitment of 53BP1 at DSBs. 53BP1 is important for the ATM-dependent phosphorylation of the chk2 kinase [20,21]. Significantly, phosphorylation of chk2 was impaired in the absence of mH2A1 (Fig. 2D), despite the observation that ATM's intrinsic kinase activity was unaltered by mH2A1 depletion (Fig. 2B). This is consistent with impaired recruitment of 53BP1 in the absence of mH2A1 leading to a defect in the 53BP1-mediated phosphorylation of chk2 by the ATM kinase.

Histone variants such as mH2A1 can be exchanged onto nucleosomes [7], suggesting that mH2A1 may be exchanged onto chromatin in response to DSBs. To explore this hypothesis, we used a designer zinc finger nuclease to create a targeted DSB [22]. p84-ZFN creates a single DSB on chromosome 19 and evokes a DSB damage response which can be monitored using standard ChIP techniques [3]. To analyze mH2A1 by ChIP, we first established stable cell lines expressing tagged myc-HA-mH2A1.1 or myc-HA-

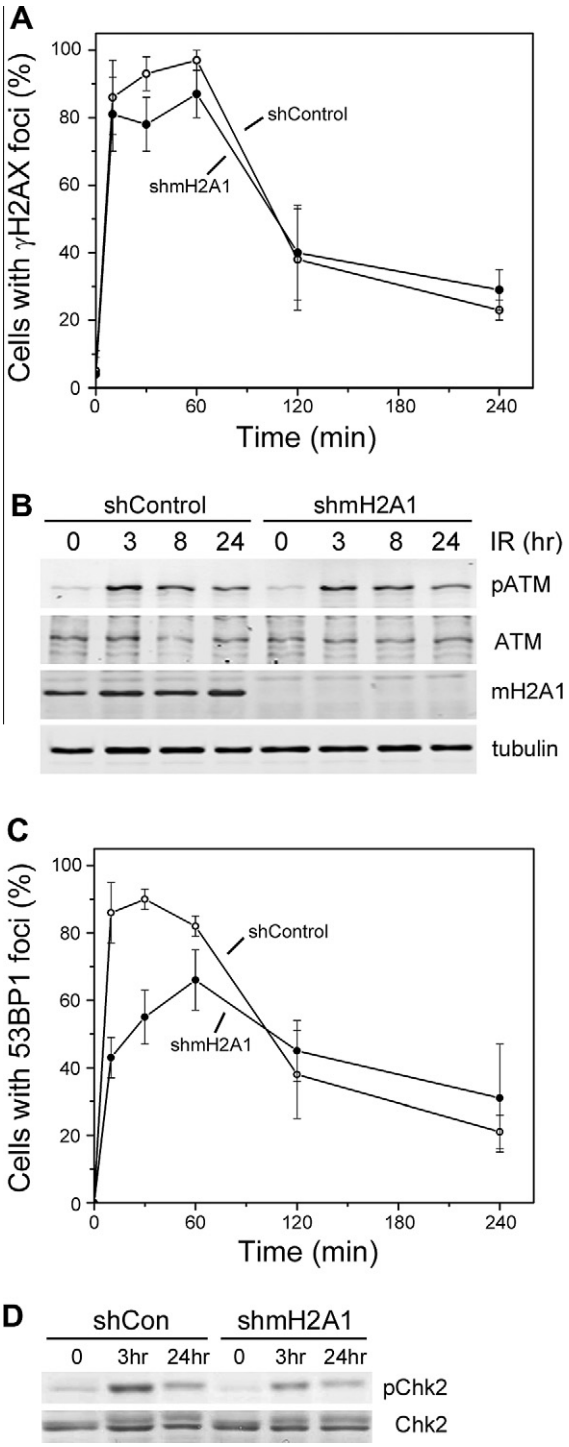

Fig. 2. mH2A1 is required for recruitment of 53BP1 to DSBs. (A) Cells expressing control shRNA or shRNA targeting mH2A1 were irradiated (2 Gy), fixed and processed for immunofluorescent staining using γH2AX antibody. The number of cells with > 5 foci were counted. Results ± SD (n > 150 cells). (B) Cells expressing control shRNA or shRNA targeting mH2A1 were irradiated (10 Gy) and analyzed by western blot with antibodies to phosphoserine 1981 of ATM (pATM), ATM, mH2A1 and tubulin (loading control). (C) Cells expressing control shRNA or shRNA targeting mH2A1 were irradiated (2 Gy), fixed and processed for immunofluorescent staining using 53BP1 antibody. The number of cells with > 5 foci were counted. Results ± SD (n > 150 cells). (D) Cells expressing control shRNA or shRNA targeting mH2A1 were irradiated (10 Gy) followed by western blot analysis with antibodies specific for chk2 and phospho-chk2.

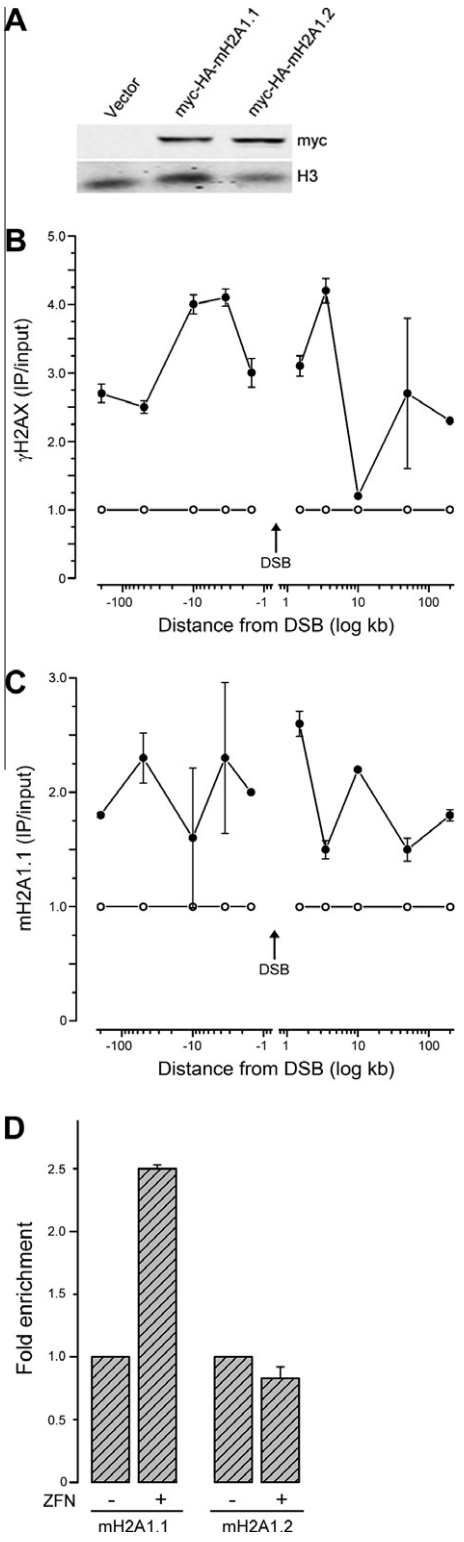

Fig. 3. mH2A1.1 is recruited to DSBs. (A) Expression of myc-HA-mH2A1.1 and myc-HA-mH2A1.2 in 293T cells. (B) and (C): 293T cells expressing myc-HA-mH2A1.1 were transiently transfected with vector or p84-ZFN. 18 h later cells were processed for ChIP analysis using either (B) γH2AX antibody or (C) HA antibody to detect the HA-mH2A1.1. Samples were processed for ChIP using the indicated primer pairs. ChIP data points were calculated as IP DNA/input DNA and were normalized to the uncut sample, which was assigned a value of 1. Results ± SD ($n = 2$). (D) 293T cells expressing myc-HA-mH2A1.1 or myc-HA-mH2A1.2 were transiently transfected with vector or p84-ZFN. 18 h later cells were processed for ChIP analysis using HA antibody as described above.

mH2A1.2 (Fig. 3A). ChIP using γH2AX antibody demonstrated that DSBs created by p84-ZFN create γH2AX domains which extend at least 200 kb from the break (Fig. 3B). Further, ChIP analysis revealed that HA-mH2A1.1 accumulated at the DSB (Fig. 3C), forming domains which were contiguous with those for γH2AX (Fig. 3B). However, ChIP analysis failed to detect any significant accumulation of mH2A1.2 at the DSB (Fig. 3D), indicating that only the mH2A1.1 splice variant is recruited to sites of damage.

Recruitment of mH2A1.1 to DSBs could reflect either exchange of mH2A1.1 onto nucleosomes or increased association of mH2A1.1 with the damaged chromatin. During ChIP, chromatin-associated proteins require cross-linking to nucleosomes to allow immunoprecipitation of the protein-DNA complex, whereas histones, which are integral components of the nucleosome, do not require crosslinking. Fig. 4A demonstrates that γH2AX, an integral component of nucleosomes, was readily detected by ChIP in both the presence and absence of cross-linking. In contrast, recruitment of the DNA damage response protein Ku70 to DSBs was significantly decreased without prior cross-linking, consistent with Ku70 interacting with the DNA ends at DSBs. Importantly, mH2A1.1 recruitment to DSBs was significantly reduced in the absence of cross-linking (Fig. 4A), implying that mH2A1.1 is recruited to DSBs but is not exchanged onto the nucleosomes. Because the mH2A1.1 splice variant contains the PAR binding macrodomain [7], we examined if recruitment of mH2A1.1 required interaction with PAR, which is generated by PARP1 at DNA strand breaks [11]. The PARP inhibitor AZD2281 blocked recruitment of mH2A1.1 to the chromatin both adjacent to the DSB and at more distant sites (Fig. 4B). This is consistent with mH2A1.1 recruitment to DSBs occurring through interaction of its macrodomain with PAR sites at the DSB. Further, Fig. 4 indicates that mH2A1.1 is not stably incorporated into nucleosomes at DSBs.

4. Discussion

Here, we demonstrate that mH2A1 plays a central role in the DSB repair pathway. Although mH2A1 has 2 splice variants, only the mH2A1.1 variant, which contains the PAR-binding macrodomain [9,11], was recruited to DSBs. Further, mH2A1.1 was not exchanged onto nucleosomes at the DSB. Instead, retention of mH2A1.1 required PARP1 activity, implying that mH2A1.1 is recruited to DSBs through a unique mechanism involving interaction between mH2A1.1 and PAR chains created by PARP1 at DSBs [11]. This is consistent with other studies demonstrating that recombinant macrodomain modules [9] and the ALC1 remodeling complex are localized to sites of DNA damage through interaction with PAR [13].

Loss of mH2A1 led to an increase in radiosensitivity, indicating a defect in DSB repair. However, we also observed an increase in NHEJ activity and a delay in the recruitment of 53BP1 to breaks in the absence of mH2A1. 53BP1 has been implicated in regulating NHEJ through its role in mediating long range interactions required for end-joining [19] and to function as an inhibitor of homologous recombination [18]. However, the role of 53BP1 in NHEJ is poorly defined, and the current work does not allow us to determine if the increased NHEJ activity in mH2A1-deficient is due to the observed delay in 53BP1 loading, or arises through disruption of other mH2A1 dependent pathways which regulate NHEJ. Nevertheless, loss of mH2A1 did lead to a significant defect in the 53BP1-dependent phosphorylation of the chk2 kinase by ATM [20,21], consistent with a key role for mH2A1 in regulating the functional activity of 53BP1.

Recruitment of mH2A1.1 to DSBs may have several potential impacts on the DNA damage response. 53BP1 recruitment requires

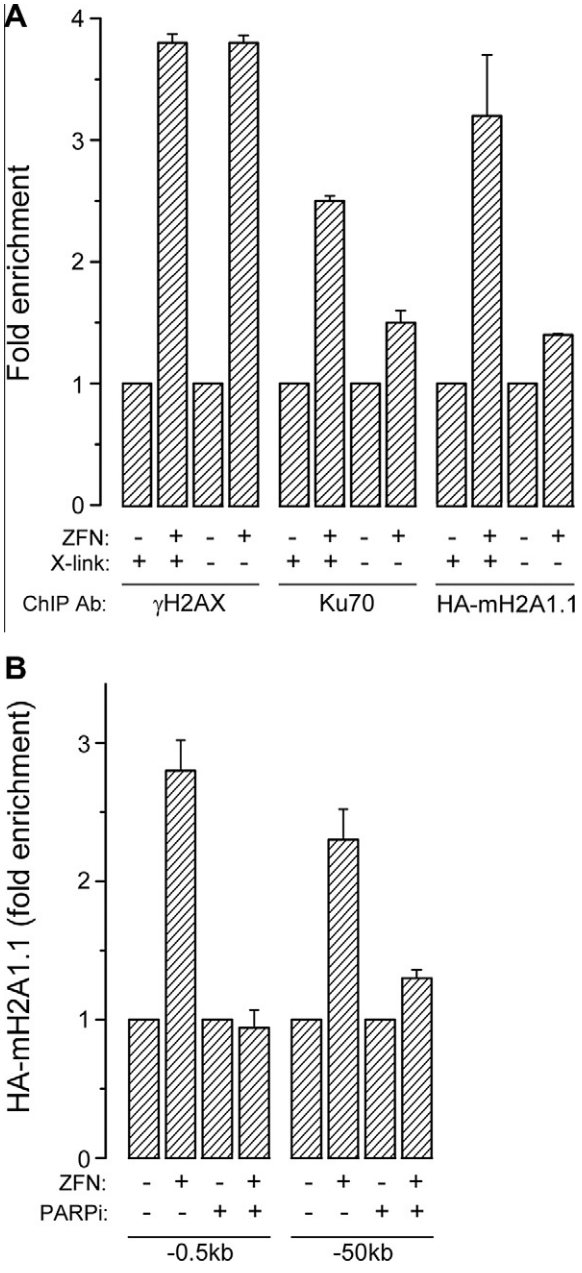

Fig. 4. mH2A1.1 is not incorporated into nucleosomes at DSBs. (A) 293T cells stably expressing myc-HA-mH2A1.1 were transfected with p84-ZFN as indicated. 18hr later, cells were either mock cross-linked or cross-linked with formaldehyde (X-link). Cells extracts were processed for ChIP using antibodies (Ab) to γH2AX, Ku70 or HA-mH2A1.1. Results ± SD ($n = 3$). (B) 293T cells expressing myc-HA-mH2A1.1 were transiently transfected with p84-ZFN in the presence or absence of PARPi (20M AZD2281). Cells were processed for ChIP and myc-HA-mH2A1.1 accumulation at the DSB (−0.5kB) or at a distant site (−50 kb) analyzed by RT-qPCR.

both chromatin ubiquitination by the RNF8/RNF168 ubiquitin ligases [23] and interaction of 53BP1 with histone H4 di-methylated on lysine 20 [24]. Interaction of mH2A1.1 with PAR sites may therefore promote changes in chromatin structure and/or post-translational modification which can facilitate both 53BP1 loading and DSB repair. Further, mH2A1.1 can inhibit PARP1 activity [25,26], suggesting that mH2A1.1 may inhibit PARP1 and limit chromatin PARylation. Controlling PARP1 activity is critical, since excessive PARylation can deplete NAD+ and energy pools, leading to cell death [11]. Recruitment of mH2A1.1 may therefore also provide a mechanism for sensing the concentration of PAR in the cell and provide a regulatory framework to control PARP1 activity and PAR levels during DNA repair.

In conclusion, our work demonstrates that recruitment of mH2A1.1 to sites of damage without its subsequent exchange onto nucleosomes plays a critical role in DSB repair and 53BP1 function. Further, this work underscores the importance of macrodomains and PARylation of the chromatin in the detection and repair of DSBs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.030.

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